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The Transformational Role of Polymerase Chain Reaction (PCR) in Environmental Health Research

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Abstract
The Polymerase chain reaction (PCR) has transformed the entire world of scientific research, especially the biological sciences and its related disciplines. It is a technique that allows the exponential amplification of target DNA sequences. Polymerase chain reaction has tremendously wide application in diverse fields of study. Some of these include, Classification of organisms, Archaeological studies, Mutation detection, DNA Sequencing and phylogenetic studies, Diagnosis of medical disorders and diseases, Cancer research, HIV detection, Detection of pathogens (both plant and animals), Genetic fingerprinting, Drug discovery and toxicity testing, Genetic engineering, Pre-natal diagnosis of genetic disorders, Environmental pollution monitoring and control, Environmental and public health monitoring, forensic and criminology, etc. Whilst, the use of Polymerase chain reaction procedures are widely adopted by industries, laboratories and research centres in developed countries, developing countries such as Nigeria are yet to harness the tremendous benefit of this revolutionary scientific tool of molecular biology. Hence this paper attempts to elucidate the usefulness of PCR-based research as it relates to Environmental health in particular. Unlike the traditional use of culture media in isolation and detection of pathogenic organisms in environmental samples, which are often fastidious, slow and sometimes grossly misleading, the use of PCR-based molecular approach is rapid and sensitive and could be used as an alternative method for monitoring environmental health quality.
Keywords: PCR, Molecular Biology, Environmental health, DNA

Introduction

Although the exact impact of environmental factors on disease and death is not fully understood, studies have shown that human health is largely dependent on the environment (Esery *et al.,* 1991; Baltazar, 1988; Remoundou and Koundouri, 2009). In particular, the World Health Organisation (WHO) in one of her reports indicated that preventable environmental factors account for about thirteen million deaths annually worldwide (WHO, 2006). Also in that report, the World Health Organization specifically disclosed that 24% of the global disease burden (healthy life years lost) and 23% of all deaths (premature mortality) are attributable to environmental factors. Diarrhoeal diseases topped the list of disease with the largest absolute burden attributable to modifiable environmental factors. Others were lower respiratory infections, 'other' unintentional injuries and malaria.

In an earlier report, the World Health Organization (WHO) had estimated that 2.2 million people die annually from diarrhoeal diseases and that 10% of the population of the developing world are severely infected with human pathogens related to improper waste and excreta management (Murray and Lopez 1996; WHO 2000). Sadly, though expectedly, the incidence of disease on account of environmental factors is far much higher in developing countries than in developed countries, due to differences in exposure to environmental risks and access to quality health care (WHO, 2006).

Majority of the Nigerian population resides in sub urban and rural settlements with high risk for environmental health hazards. Exposure to some of the pathogenic microorganisms which form integral communities of such environments are known to cause a number of human diseases, such as diarrhoea, cholera, dysentery, typhoid, malaria anthrax, tuberculosis, aspergillosis, schistosomiasis, ascariasis, polio, cryptosporidiosis etc. Sometimes even normally innocuous microorganisms and their metabolites may lead to disease (Yang, 2004), especially in people with compromised immune system. This calls for diagnostic methods that would detect and identify microorganisms within our environment, and in diseased patients. Such methods must be fast and reliable to ensure accurate assessment of microbial contamination of the environment, which in turn would facilitate the initiation and implementation of a remediation process (Yang, 2004).

Prior to the advent of the PCR technology, the sampling and testing for microorganisms had depended solely on traditional use of culture media. The use of culture media in investigating environmental samples is not only cumbersome and slow, but it is highly unreliable owing to the fact that most microorganisms are not culturable, some, especially fungi are fastidious and very plastic making their identification rather speculative (Etebu, 2008). In contrast, PCR-based molecular approaches offer a rapid, simple and reliable ways to detect and quantify biomarkers of health hazards in the environment without recourse to culture media (Etebu, 2009).

Although the use of Polymerase chain reaction procedures are widely adopted by industries, laboratories and research centres in developed countries (Dong *et al.,* 2008), developing countries such as Nigeria are yet to harness the tremendous benefit of this revolutionary scientific tool of molecular biology. This lackadaisical posture observed in Nigeria towards the use of PCR could, in part, be due to the huge dearth of knowledge on the manifold uses and applications of this scientific innovation. Hence this paper attempts to elucidate the procedures and usefulness of PCR-based research as it relates to Environmental health in particular. The write up is intended to generate interest in PCR among academia and government functionaries saddled with the responsibility of policy formulations and governance.

The basis and mechanics of PCR

The Polymerase chain reaction stems from our knowledge in *in vivo* DNA replication. DNA replication is central to all of biological science. An essential property of the genetic material is its ability to provide for exact copies of itself. A DNA replication is a process that occurs only once in each cell generation, and is the most important event in the replication of chromosomes, and depending on the cell involved, it ultimately leads to either mitosis or meiosis.

DNA replication occurs very rapidly; rate of synthesis is about 50 nucleotides per second in humans and about 500 nucleotides per second in prokaryotes. DNA replication begins with 'unzipping' of the DNA molecule. The DNA molecule 'unzips' with the paired bases separating as the H_2 bonds are broken; leading to two single DNA strands. As single strands, each directs the synthesis of two new complementary strands along its length in the 5'-3' direction as revealed in the semi-conservation model and proven in the density transfer experiment of Meselson and Stahl (Alberts, *et.al.*, 2002; Griffiths *et al.,* 2008).

DNA replication begins with the unzipping or unwinding of the double helix at a site with a specific nucleotides sequence called the origin of replication. A protein, the initiator proteins recognizes and binds to this site (origin of replication). This union between DNA and initiator protein attracts an enzyme called DNA helicase which breaks the H_2 bonds linking the adjacent complementary bases to open up a region of DNA at the Origin of replication. The opening of this region of DNA creates two Y-shaped areas or Replication Bubble. Each of the Y-shaped areas is called a replication fork (Griffiths *et al.,* 2008).

Single-stranded binding proteins ensure that the two strands of a replication fork remain separated and allow the enzyme (DNA Polymerases) necessary for synthesis to bind, and DNA polymerase adds nucleotides, one after the other, to the end of a growing DNA strand. Suffice to state that each new strand of DNA begins with a short RNA primer formed by the enzyme RNA primase (Griffiths *et al.,* 2008; McCulloch *et al.*, 2008). As DNA replication proceeds, helicase progressively unwinds the double helix to expose successive nucleotide sequences. This enables DNA polymerase to add nucleotides continuously to the growing 3' end of the new strand called the Leading strand, while the other strand, the Lagging strand is synthesized in bits or fragments, called Okazaki fragments, though still in the 5'-3' direction. As the Okazaki fragments lengthen they are ultimately joined together by the enzyme DNA ligase. Prior to the joining of the Okazaki fragments, the RNA primers are replaced with DNA sequences in both the Leading and Lagging strands of newly synthesized DNA.

The Polymerase chain reaction stems from our knowledge in *in vivo* DNA replication described above. It is more or less *in vitro* DNA replication. The polymerase chain reaction (PCR) is a technique that allows the exponential amplification of target DNA sequences (Saiki *et al*., 1988). It involves the use of a pair of single stranded short oligonucleotides (or primers) usually about 18-30bp which are complementary to a known gene sequence in bulk DNA sequences (Etebu, 2008).

PCR amplification is characterised by a sequence of three processes; the first process entails a denaturing of the double stranded DNA template into single stranded DNA at a high temperature (>90^{o}C). This is followed by the annealing stage often considered to be the most critical stage. At this stage, the primers bind to complementary sequences on the template DNA. Following the annealing stage is the extension of bound primers by a thermostable DNA polymerase enzyme (*Taq*) via a process in which the enzyme uses the deoxynucleoside-5'-triphosphate (dNTP) molecules available in the reaction matrix to build an oligonucleotide chain complementary to the template DNA, forming a double stranded DNA molecule again (Saiki *et al*., 1988).

The application of PCR in detecting and investigating Environmental health biomarkers in the environment is basically centred on the use of species-specific oligonucleotide primers which hybridise with DNA extracted from a sample under investigation. These primers can be labelled depending on the specific method concerned, and they facilitate the selective amplification of specific region(s) in the genome of the organism(s) of interest. Polymerase Chain Reaction (PCR) could duplicate a given sequence of DNA over several million-fold within a relatively short time (Mullis and Faloona, 1987; Saiki *et al*., 1988).

After PCR, the amplified sequences are resolved in an electrophoresis gel, usually made of agarose or polyacrylamide gel (Brock *et al*., 1994; Kainz *et al*., 1992). Electrophoresis allows the movement of charged molecules in electric field, the rate of movement being dependent on size, electrical charge (Brock *et al*., 1994)

and in some cases, sequence variability as in Denaturing gradient gel electrophoresis (DGGE), Temperature gradient gel electrophoresis (TGGE) etc (Muyzer *et al*., 1993; O'Callaghan *et al*., 2003). Electrophoresis indicates the fragment size of the amplified products (Nicholl 2002).
With some experiments, PCR, followed by resolution of the amplified DNA products via gel electrophoresis is enough to derive useful information to that experimental hypothesis. Whereas for some others, a variety of procedures are employed to further analyse the products. For example, experiments aimed at assessing the phylogenetic relationships or diversity of genetic profiles within an environmental sample-DNA would require additional molecular approach such as DGGE, Terminal restriction fragment length polymorphism (T-RFLP) etc (Suga *et al*., 2000; Muyzer *et al*., 1993; Etebu, 2013). However, endpoint PCR would be used first to confirm the suitability of relevant primers to target and amplify the gene sequence of interest.

Setting up Polymerase chain reaction
To set up a typical PCR, certain chemicals and reagents has to be pooled together to form the PCR mix. A typical standard PCR mix requires a DNA template containing the region to be amplified. This could be crude cell extracts, total genomic DNA, Plasmid DNA etc (ng quantities) Aside the DNA template, a number of other constituents shown in Table 1 are also required.
As simple as the PCR process appears to be it is imperative that certain steps be given due attention. Such steps and the required basic equipment are hereunder highlighted.

1. Primer design
- The primers should not be Complementary at their 3' ends
- Avoid possibility of hairpin formation (2° structure)
- Melting temperature (T_m) of both primer should be about the same; $T_m = 2*(A + T) + 4*(G + C)$
- Primer should be as specific as possible (use BLAST Analysis to ascertain potential specificity)
- Primers should not anneal to themselves

2. Annealing temperature
3. Preventing contamination
4. Choice of polymerase
5. Magnessium concentration
6. Number of PCR cycles
7. Choice of equipment (See fig. 2)

(Adapted from Wilfred *et al.,* 2005; Etebu, 2008)

Applications of PCR in Environmental Health Investigation and Research
Majority of the Nigerian population are said to reside in sub urban and rural settlements with high risk for environmental health hazards as those shown in figure 3. Environments such as those shown in this figure would commonly be infested with diarrhoea related human pathogens. The transmission of many infectious diseases has been linked to environments polluted with human excreta harbouring pathogenic microorganisms that cause the diseases. Disease conditions are exasperated by the lack of adequate personal and domestic hygienic practices among those living in such environments (Carr, 2001). Faecal-oral infections caused by bacteria, virus, helminthes and protozoa linked to unsafe water and lack of hygienic sanitary practices are a great source of concern; causing huge burden of infectious diseases like diarrhoea, cholera, typhoid, enteric fever, hepatitis and critically high infant mortality.
Environmental investigations using PCR technology is best used to track specific organisms (Yang, 2004). PCR technology has become an essential research and diagnostic tool for improving Environmental, human health and quality of life because it allows scientists to detect the presence or absence of a specific virus, bacterium or any particular sequence of genetic material from any environment, be it air, water or soil (Alvarez *et al.,* 1995; Makino *et al.,* 2001; Bielawska-Drózd *et al.,* 2012).
Polymerase chain reaction techniques have been used in experimental research work over the years to investigate various diseases and disease causing agents of relevance to environmental health. Some of the diseases are presented in Table 3 and includes amongst others, anthrax, cholera, typhoid, dysentery, schistosomiasis, ascariasis, cryptosporidiosis, malaria, hepatitis, and polio

Limitations of Polymerase chain reaction
Like every other human endeavour, PCR is confronted with a couple of limitations. Accurate characterization or identification of microorganisms by PCR is dependent on nucleic acid sequences isolated from an environment or sample under investigation. So in essence, PCR is influenced by the same bias and variations that are inherent in many nucleic acid extraction techniques (Milling *et al.,* 2005). Another often contentious limitation of PCR

analysis of environmental samples is the inhibition of the enzymatic reaction. Whereas humic substances are known to inhibit the DNA polymerase enzyme, colloidal matter has a high affinity for DNA. The presence of these contaminants in a sample can therefore substantially affect the amplification yield of PCR product.

Conclusion

Limitations notwithstanding, the polymerase chain reaction technique has proven to be very useful in environmental health research. Firstly, the PCR technology has been successfully applied to monitor the environment to detect and sometimes quantify specific microorganism of health importance. For example, PCR could be used to monitor hospitals and public conveniences over time for incidence of pathogens relevant to public health.

Secondly, PCR could also be used to trace the source of pathogenic inoculum. This could very well be done by using appropriate primers in a polymerase chain reaction. In doing this, strain-specific primers must be used if the organisms of interest are common in the environment, or better still, pathogenicity genes inherent in pathogenic forms of microorganisms could be targeted as exemplified in the innovative works of Etebu and Osborn (2009, 2010, 2011a-d) with the pea footrot disease pathogen, *Nectria haematococca*.

Thirdly, PCR could be applied to study the microbial ecology of specific environments or reservoirs. PCR methods are commonly used in water quality studies and for evaluation of biofilms. In addition, the PCR technique has been used extensively to study microbial community diversities. The same principle could also be used to study the genetic diversity and phylogenetic relationship between different microorganisms within an environment or those implicated together in a diseased condition. Microbial communities have often been studied targeting the ribosomal rRNA genes (16S or 23S for prokaryotes and 18S or 28S for eukaryotes). This type of investigation has been done with respect to two species of *Ascaris* (Cavallero *et al.,* 2013)

The application of PCR technology in environmental investigations and research has proved to be a viable and more reliable alternative to traditional methods involving culture medium or serology. It has indeed revolutionized not just environmental health research but the entire field of pure and applied biology and its related disciplines such as agriculture, medicine, environmental sciences etc. It would do Nigeria a great deal of good if the academia, health and other sectors in the country utilize this revolutionary scientific technology in her transformational agenda.

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Table 1: PCR Reaction Components for 1 Reaction mix (Adapted from: Etebu, 2008)

Components	Concentration/volume required
Forward primer (18-30bp)	20pmol (1-2µl)
Reverse primer (18-30bp)	20pmol (1-2µl)
Amplification buffer	One-tenth of final volume (5µl)
dNTP	20-200µM each of dATP, dCTP, dGTP & dTTP
$MgCl_2$	50mM (1.25µl)
Taq polymerase	1µl (1U)
Template DNA	10-100ng
Sterile distilled H_2O	Make up to 50µl

Table 2: Some environmental health agents detected through the use of PCR technology

S/No.	Disease	Target agents/ organism(s)	Target gene(s)	Reference(s)
1	Anthrax	*Bacillus anthracis*	*Cap*	Makino *et al.,* 1989, 2001
2	Cholera	*Vibrio cholerae*	*ompW, ompU, ctxA and zot*	Bielawska-Drózd *et al.,* 2012
3	Typhoid	*Salmonella typhi*	Flagellin (H-Id)	Ambati *et al.,* 2007
4	Dysentery	*Shigella* spp.	Plasmid antigen (ipa)H	Islam *et al.,* 1998
5	Schistosomiasis	*Schistosoma mansoni* and *S. haematobium*	Cytochrome C oxidase	ten Hove *et al.,* 2008
6	Ascariasis	*Ascaris lumbricoides* and *A. suum*	ITS rDNA	Cavallero *et al.,* 2013
7	Cryptosporidiosis	*Cryptosporidium* spp.	18S rRNA	Coupe *et al.,* 2005
8	Malaria	*Plasmodium* spp.	18S rRNA	Johnson *et al.,* 2006
9	Hepatitis	Hepatitis virus	5' non coding region of HCV RNA region	Farma *et al.,* 1996
10	Polio	Polio virus	VP1-2A region of the poliovirus genome	Chezzi, 1996

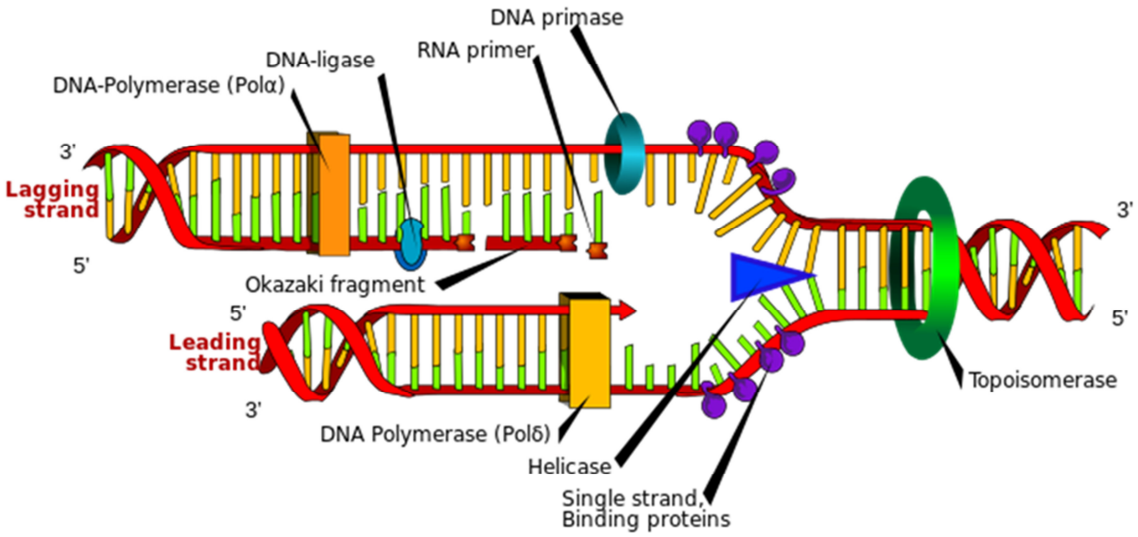

Fig. 1: Schematic diagram of *In vivo* DNA replication (Source: www.Images.google. Com)

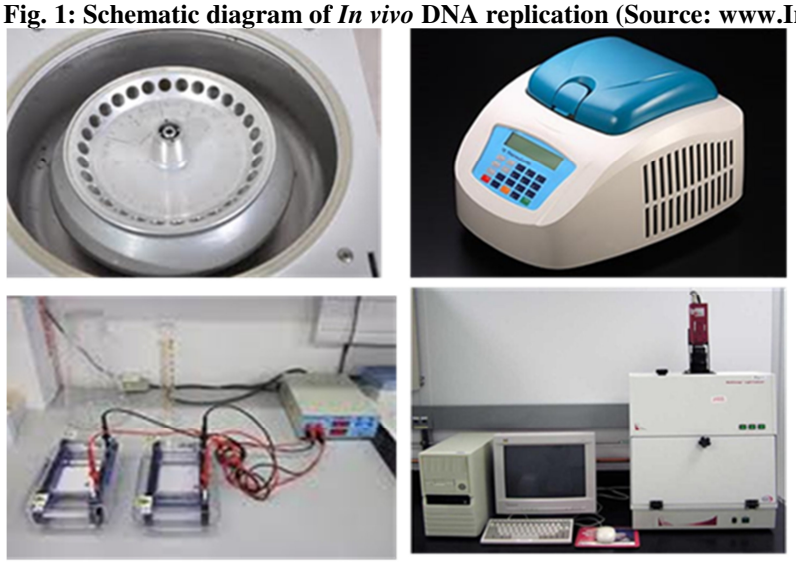

Fig. 2: Some essential equipment required for a standard PCR (Source: www.images. Google.com

Fig. 3: A typical living environment in Yenagoa metropolis, Bayelsa State, Nigeria